AN IMPROVED METHOD OF ISOLATING BRUCELLOSIS ORGANISMS FROM BLOOD CLOTS UTILIZING THE MEMBRANE FILTER

by

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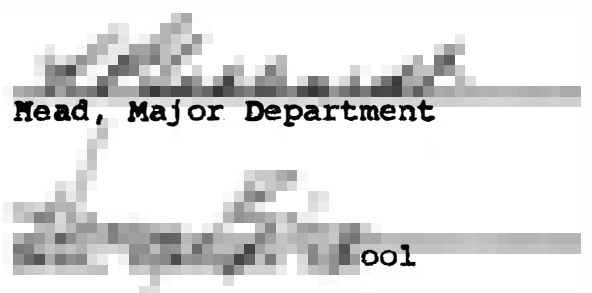

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I. INTRODUCTION

The origin of the disease which today we term brucellosis is lost in time. Only since the end of the last century has this infection been described and set apart as a separate disease entity from typhoid, malaria, and other febrile diseases.

The deceiving nature of this disease helped to create confusion in the positive diagnosis of brucellosis. It was difficult to isolate the causative agent and transmission from animals to man was questionable.

Because of this "dogged resistance" of the organism to easy isolation from blood and tissues, clinicians sought diagnostic aids in the agglutination reaction and other clinical tests such as the opsonocytophagic index and the allergic skin test. These tests could only tell the clinician that the disease was either a past experience with the patient or that it might be a chronic infection. For active or acute brucellosis, isolation of this bacillus from the patient was the only reliable available method for diagnosis of the disease.

The isolation of Brucella species from blood clots has been a source of concern to laboratory workers because of the insensitivity of the several different isolation methods. The design of this research, therefore, was to develop a

method of *Brucellae* isolation from blood clots that would increase the percent of isolation when only a few organisms are present, be economical and easy to perform, and shorten the time factor in laboratory diagnosis.

The membrane filter was chosen as the chief method for the recovery of organisms from blood clots for several reasons: (a) The filter retains all organisms during filtration and provides an improved and economical method for culturing and incubating the deposited organisms when the under surface of the filter disc is brought into contact with appropriate nutrients; (b) The organisms retained on the surface of the filter are separated from soluble enzymes or inhibiting substances which may have been present in their previous environment; and (c) The uniform size and frequency of pores in the filter provide rapid filtration of liquids.

II. LITERATURE REVIEW

Sir David Bruce (1) is considered the first to have successfully cultivated Brucella melitensis on agar media, having succeeded in culturing a "micrococcus" from the spleens of four fatal cases of Malta fever. His observations were published in a short paper in 1887. He successfully transmitted the disease to monkeys and designated the disease as Mediterranean fever and named the etiological agent Micrococcus melitensis (2).

In 1895, Professor Bang (3), a Danish veterinarian, having purchased a cow that was aborting, removed the calf and the membranes. Using a medium of serum and gelatin agar Bang isolated, in pure culture, an organism he designated as a bacillus. He found that growth occurred at two optimal concentrations of oxygen, one was just below the content of atmospheric air, the other about 90 percent of air, and that poor or no growth took place between these two concentrations.

It should be mentioned that Bruce had described coccoid forms for his microbe and had designated it as a micrococcus. Furthermore, his organisms reproduced readily on the surface of culture media. Bang, on the other hand, described his bacterium as a bacillus, the growth of which did not take place under atmospheric conditions, but only in reduced concentrations of oxygen. These distinctions were to prove stumbling blocks for subsequent workers, and to help conceal

the kinship of the two species of micro-organisms which went unrecognized for many years.

Bacteriologists were shocked when Alice Evans (4) announced in 1917 that she had compared the so-called Micrococcus melitensis with Bang's organism of contagious abortion and found a close resemblance between them, not only in morphology, but also in cultural and serologic behavior. Unqualified proof for Evans' assumption was given by Orpen (5) in 1924 when he showed that Br. abortus does cause human disease. His studies on strains isolated from patients who had never ingested goats' milk but had had close association with infected cattle showed them to have the disease undulant fever.

Ever since Wright (6) first introduced the agglutination test in 1887, it has remained, according to Spink (7), the most dependable single test in the diagnosis of brucellosis. Spink (7) qualifies this by stating that isolating the organisms from the tissue and body fluids constitutes the most reliable diagnostic evidence, but cultural studies often yield no Brucellae in the face of obvious active disease. The isolation of Brucellae from the blood, however, has become the prime objective of many laboratories because of the inconclusiveness of other laboratory tests.

Freshly-drawn citrated blood, as suggested by Pickett and Nelson (8) seems to offer the best chance of isolation but a clotted sample (submitted usually for agglutination

studies) is the type most frequently submitted by the physician to a public health laboratory.

The cultural methods employed by Borman and West (9) in the isolation of Brucellae from blood clots were methods that were almost universally used until 1947. First the serum was removed and the clot was broken up by means of a sterile glass rod, or else forced through a 5 ml syringe without a needle. The next step was to inoculate the broken clot material into tryptose broth containing a 1×10^{-6} dilution of crystal violet and incubate under a 10 percent carbon dioxide and air mixture. Every seven days for three weeks subcultures were made from the broth to crystal violet tryptose agar plates, which in turn were incubated four days under 10 percent carbon dioxide and then checked for Brucellae colonies.

A rather simple but unique modification of this method is the now famous (introduced in 1947) Castaneda (10) double media technique. This procedure eliminates the need for subculturing from broth to agar because the two are contained in one unit. The tryptose agar is solidified on one of the sidewalls of a 4-ounce prescription-type bottle and then about 30 ml of sterile tryptose broth is added to the bottle in an upright position. The unit can be stoppered with a rubber vaccine bottle stopper and then 10 percent carbon dioxide added by needle. This type of stopper is only satisfactory for fresh or citrated blood as it is virtually impos-

sible to push much of a clot through a needle. Once the blood is added to the broth and incubated for a day or two, then the agar slope in the bottle can be seeded from this broth (my merely tilting the bottle enough to wet down the slope) and reincubated in an upright position. If colonies appear on the agar slope, these can be subsequently identified by appropriate methods. If there are no colonies then the bottle is tipped again to wet the slope and reincubated. The Castaneda method offered two advantages: it prevented contamination which might occur following multiple subculturing and it helped protect laboratory personnel against the risk of infection.

A method which utilized the yolk sac of 3 to 5 day old chick embryos for primary isolation of Brucellae was published by Damon (11) in 1951. The idea that Brucellae would grow well in chick embryos was not new; Goodpasture and Anderson (12) in 1937 and other subsequent investigators (13,14) had described this organism as preferentially an intracellular parasite. In Damon's method the blood clot was mascerated through a syringe into a bottle containing tryptose broth and glass beads. The bottle was then shaken for three minutes to further disintegrate the clot. One-half ml of this broth-clot mixture was then inoculated into the yolk sac of the 3 to 5 day old chick embryo. Two or three subcultures are made at 4 to 5 day intervals with yolk material onto the surface of a slightly dehydrated tryptose agar plate.

Damon (15) made a comparative study in 1952 utilizing the above technique, guinea pigs for isolation, and the routinely used crystal violet tryptose broth method. Of the three methods the directly inoculated guinea pigs was the least effective giving a 1.1 percent isolation. The chick embryo gave a 12.6 percent isolation compared to 6.3 percent for the crystal violet tryptose broth method. All three methods were compared by inoculating from the same clot into each media as well as into animals.

The membrane filter technique as a method for cultivating bacteria was first introduced into the literature of this country in 1951 by Goetz and Tsuneishi (16). Their method was designed as a new and faster way for the bacteriological analysis of water. Kabler and Clark (17) followed suit in adapting the filter to differential media. Their use of the filter was also along sanitary bacteriology lines with experimental suggestions as to how it might be used with pathogenic enteric micro-organisms.

The first to successfully cultivate a fastidious organism on the membrane filter were Braun and Kelsh (18) in 1954 at Camp Dietrick, Md. They grew Br. suis on the membrane by the passage of a previously laked 1 ml aliquot of heparinized rabbit blood. The blood was drawn from an artificially infected rabbit and the cells were laked with distilled water. They placed their membranes on an agar medium base to incubate (distinguishing this method from Goetz's (16) absorbent pads).

If Brucella cells were present on the surface of the membrane filter, these would develop into visible colonies within 3 to 4 days. Braun and Kelsh made a comparative study of their membrane filter method with the popular Castaneda (10) double media method. They indicated that their method of Brucella isolation was as good and probably superior to the Castaneda method and that their filter technique appears to permit a quantitative estimation of the extent of bacteremia.

In defense of their method, Braun and Kelsh point out that it is unfortunate that present methods of Brucella isolation from blood (namely Castaneda's) suffer from the disadvantage that liquid media cultivation cannot be eliminated because of the relative scarcity of the organisms in the blood. Such cultivation in liquid media even for brief periods is undesirable because: (a) Certain lots of a widely used liquid media, tryptose broth, have been shown to contain antibrucellar substances (19), and (b) Cultivation in liquid media, particularly in the presence of a patient's antiserum, promotes rapid population changes that may lead to the establishment of varient types differing considerably from the type or types originally present in vivo (20).

Tidwell and Gee (21) in 1955 reported on their experiments with the membrane filter and its use with blood cultures. For the most part their method is the same as Braun and Kelsh's except that 2 rather than 1 ml of heparinized rabbit blood was used. The infected animal red cells were laked by

repeated washings with sodium citrate instead of with distilled water. They used a variety of enteric bacteria, streptococci, and staphylococci for their test organisms to artificially infect their rabbits. Instead of placing their membranes on an agar medium, they used the original absorbent pad method (16) and their pads were soaked in double strength Albimi Brucella broth. Like Braun, they ran all their blood samples in duplicate, using a routine blood culture method for comparison with the membrane method. They claim that isolation of bacteria using the membrane filter to be as good or better than the routine blood culture method.

Spink (22) has reported that a modification of the Castaneda double media method is used in the clinics and hospitals of the University of Minnesota. This variation amounts to a modified Albimi Brucella agar, and broth substituting for the tryptose agar and broth. Some additional agar and sodium citrate were added to the Albimi agar and the broth was fortified with more dextrose and sodium citrate. Spink also states that trypticase-soy broth (Baltimore Biological Laboratories) and agar were found to be highly satisfactory in quantitative studies carried out with several different media.

III. EXPERIMENTAL METHODS AND MATERIALS

A. Test Organism.

An avirulent stock culture strain #456 of Brucella abortus was used throughout these studies. It has been used in the preparation of antigen for the Brucella agglutination test in the Utah State Health Laboratories for several years. Fresh 72-hour subcultures of this organism were used in all experiments.

B. Growth Media.

Pork and beef liver infusion agars and broths were used in all experiments. They were prepared at a ratio of 1 lb. of fresh liver that had been ground and infused with 500 ml of distilled water. A commercial medium, B.B.L.'s trypticase-soy agar and broth was also used in these experiments. The composition of the liver infusion broth was as follows:

(per 100 ml)

Liver infusion	50 ml
Peptone (Difco)	1.0 gm
NaCl	0.5 gm
Dist. water	50 ml

Difco Bacto agar in both 2 percent and 1 percent concentrations was added to the above to prepare the agar infusions. Crystal violet (23) was added to these media in later experiments to give a final concentration of 1×10^{-6}). The pork and beef liver infusion broths were prepared in both single (as given above) and double strength concentrations.

The pH of the infusions was adjusted to 7.0 prior to sterilization. All media were sterilized in the autoclave at 15 pounds pressure for 15 minutes.

C. Equipment.

The equipment sterilized in the autoclave sterilizer for use in these experiments included: the Millipore membrane filter holder and suction flask; membrane filter discs and absorbent pads; flat-bladed forceps; 5 cm petri dishes; normal (0.85 percent) saline; distilled water, sand and glass beads, and desicator jars used for CO_2 tension.

D. Enzyme Preparation.

Streptokinase*, a commercial enzyme preparation, was used in a concentration of 1000 units per ml. The method of use was patterned after that of Watson (24) who indicated that the successful clinical use of the fibrinolytic streptococcal enzyme suggested that it might prove of value in typhoid clot cultures. The enzyme itself was unexpectedly encountered by Tillett and Garner (25) in 1933 while conducting other studies that involved plasma, fibrinogen and streptococci. Later, Christensen (26) did studies directed toward purifying and isolating the streptococcal fibrinolytic principle in order to investigate its essential nature.

E. Growth of Test Organism on Membrane Filter.

The intent of the first preliminary experiments was to

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* Varidase-product of Lederle Lab., Div. of Amer. Cyanamid Co. Supplied in 20,000 units (white powder): 15,000 units streptokinase, 5,000 units streptodornase.

determine if a given strain of Brucella would grow on the surface of a membrane filter disc. The methods of Goetz (16) and Kruse (27), which had been developed for the enumeration of coliform organisms in water, were used for this series of experiments.

1. Method of Goetz: A loopful of the test organism grown on a single strength pork liver infusion agar slant was suspended in 10 ml of sterile saline. About 2 ml of this suspension were filtered through a membrane filter. The filter was then transferred to a sterile absorbent pad saturated with sterile double strength liver infusion broth.

2. Method of Kruse: The use of the filter assembly for this method is the same as in the Goetz method. The difference lies in the method of supplying nutrients to the membrane filter. Instead of using a saturated nutrient pad, Kruse merely places the filter disc on a prepoured agar plate (in this case a 5 cm petri dish) containing nutrients in double strength.

F. Double Versus Single Strength Infusion Agars.

It was of interest to know if this Brucella strain would require double strength nutrients in the agar when using the Kruse method. Four more membrane filtrations were carried out using the Brucella-saline suspension mentioned above. Two were placed on single strength pork liver infusion agar and two on the double strength infusion agar. All of the above cultures were incubated at 37°C. under 2 percent CO_2 tension (smokeless candle method) for 48 hours.

G. Brucella Survival Time and Blood Degradation Methods.

The purpose of this first experiment was to determine the survival time of stock Brucella organisms introduced into freshly drawn rabbit blood. Secondly, to find suitable methods for breaking down both clotted and whole blood to such a state that it will pass through the membrane filter easily. Such methods for breaking down the blood should not be so severe as to kill the organisms trapped in the blood.

1. Preparation of Suspensions of Known Numbers of Brucellae: A loopful of the test organism was suspended in 10 ml of sterile saline. After mixing well, 0.1 ml was removed and added to a second tube and so on in serial fashion through three additional tubes. Control plates, to determine the actual number of organisms present in these dilutions were made by the pour plate method.

2. Addition of freshly-drawn Rabbit Blood to Bottles Containing Organisms and Anticoagulants: Five ml of freshly-drawn rabbit blood were placed in each of six screw-cap bottles. Brucella organisms (from dilutions prepared above) and a few crystals of anticoagulants had previously been added to the bottles in the following order:

	Bottle #1	#2
Anticoag.	Ammonium oxalate	Sodium citrate
Dilution	.05 ml 10^{-4} dil.	.05 ml 10^{-4} dil.
	#3	#4
Anticoag.	Ammonium citrate	No Anticoag.
Dilution	.05 ml 10^{-8} dil.	.05 ml 10^{-4} dil.
	#5	#6
Anticoag.	No anticoag.	No anticoag.
Dilution	.05 ml 10^{-6} dil.	.05 ml 10^{-8} dil.

The bottles containing rabbit blood, organisms (and anti-coagulants, where used) were mixed well and then sealed and left to stand at room temperature for 24 hours.

3. Procedures for Treating Whole and Clotted Blood:

a. Distilled Water: Five ml of sterile distilled water were added to bottles #1 (10^{-4}), 2 (10^{-4}), and 3 (10^{-8}) each containing whole blood, and to bottle #4 (10^{-4}) containing clotted blood. The four samples were then shaken on a mechanical shaker for 10 minutes. This shaking in distilled water did not alter the clotted sample which clung to the bottom of the bottle. The three whole blood samples (which appeared hemolyzed) were then centrifuged at 1000 r.p.m. for two minutes. The supernatant fluids from bottles containing the 10^{-4}, 10^{-4}, and 10^{-8} dilutions were placed on individual membrane filters in the filter holder while negative pressure was being applied with a water tap suction pump.

b. Dilute Acids: The purpose of this procedure was to try 0.1 N. HCl and 3 percent acetic acid as a means of altering the 5 ml of clotted blood in bottles #5 and #6 mentioned in part 2 above. The clots were first forced through a 10 ml syringe to break up the main mass of the clot. Ten ml of 0.1 N. HCl were added to bottle #6 and 10 ml of 3 percent acetic acid were added to bottle #5. The clot-acid mixtures were then rapidly shaken on a mechanical shaker for two minutes. They were next centrifuged at 1000 r.p.m. for two minutes. A few ml of the supernatants of bottles #5 and #6 were placed on membrane filters and suction applied.

c. Sand, Glass Beads, and Distilled Water: This procedure was designed to determine if abrasive agents (sand and glass beads) and distilled water would make a washed cell suspension suitable for filtration. Two to three mls of freshly-drawn rabbit blood were added to each of six bottles. Sand, glass beads, sodium citrate, and Brucella organisms had been added to the bottles previously in the following manner:

Bottle #1 Citrated beads 0.1 ml Dil. F (10^{-8})	#2 Citrated beads, sand Dil. E (10^{-6})	#3 Citrated beads Dil. E (10^{-6})
#4 Clotted beads Dil. E (10^{-6})	#5 Clotted beads Dil. E (10^{-6})	#6 Clotted beads, sand Dil. E. (10^{-6})

The organism dilutions were prepared as described above and the dilutions were called D (10^{-4}), E (10^{-6}), and F (10^{-8}). Control plates of dilutions E (10^{-6}) and F (10^{-8}) were made as before with the pour plate method. The Brucella-blood-sand-beads mixtures were mixed and then sealed and left standing at room temperature for twenty-four hours.

Ten mls of saline were added to bottle #1 (10^{-8}) containing citrated cells, and to bottle #4 (10^{-6}), a clotted sample. It was found unnecessary to break the clot up in bottle #4. The glass beads partially defibrinating the clot. After the saline was added the bottles were shaken by hand a few times to mix. The suspensions were then centrifuged at

3500 r.p.m. for 15 minutes. The supernatant fluids were removed by suction through a capillary pipette. The purpose of this saline wash of the cells was to get rid of viscous material which may have been responsible for the membrane plugging.

Twenty mls of sterile distilled water were added to the washed packed cells of bottles #1 (10^{-8}) and #4 (10^{-6}). The suspensions were then shaken on a mechanical shaker for 5 minutes. The two hemolyzed samples were centrifuged for 1 minute at 1000 r.p.m. Some of the supernatant from bottle #1 was placed on a membrane filter under negative pressure. Bottle #4 was not filtered in this experiment because it took so long to pass the supernatant of bottle #1 through the filter. Two specimens, bottles #3 (citrated) and #5 (clotted) were placed in the deep freeze to determine the effect of freezing on cells as a means of altering them for better filtration. Bottles #2 (citrated) and #6 (clotted) were saved for further experiments. All of the membranes that were used in these experiments were placed on 5 cm petri dishes containing double strength pork liver infusion agar and incubated at 37°C. in 2 percent CO_2 (candle jar).

d. Streptokinase: The purpose of this experiment was to determine if stock Brucella organisms entrapped in clotted rabbit blood could be released by streptococcal enzyme action and then reisolated on the membrane filter. It should be mentioned here that single strength beef liver

infusion agar was used instead of the double strength pork liver infusion used in previous work. The agar concentration of the medium was reduced from 2 to 1 percent and crystal violet was incorporated into the medium at a dilution of 1×10^{-6}. Appropriate controls on all phases of the experiment were also set up.

Two mls of freshly-drawn rabbit blood were added to each of 5 bottles. Before the blood could clot, 0.1 ml of 5 different Brucella dilutions (prepared as described above) was added to each of the 5 bottles and mixed. After clotting, the clots were "rimmed" with a sterile needle and the samples centrifuged at 2500 r.p.m. for 5 minutes. The sera from the 5 centrifuged bloods were removed with sterile capillary pipettes; streptokinase (1500 units) was added to each of the 5 clots. The enzyme was diluted to contain 100 units per ml by adding 15 ml of sterile trypticase-soy broth to each bottle. These clot-enzyme-broth mixtures were shaken and then placed in the incubator for 48 hours.

After 48 hours incubation at 37°C. about 5 mls of the slightly cloudy supernatant fluid from each clot-enzyme-broth mixture were drawn off with capillary pipettes and filtered through a membrane filter.

H. Isolation of Brucella from "Unknown" Human and Cow Bloods.

The object of the following studies was to determine if the experimental methods devised for use with streptokinase are applicable to the isolation of Brucella from "unknown"

human and cow blood clots in cases of suspected brucellosis and Bang's disease.

The human blood clots were obtained from the Serology Division of the State Health Laboratories. All of these bloods were submitted by the physicians of the state for the Brucella agglutination test. Only those bloods with Brucella agglutination titers of 1:25 or better were cultured. The cow blood clots were obtained from the State Dept. of Agriculture Laboratory in Provo. At this laboratory a Brucella agglutination test is performed on blood sent in by veterinarians on all Bang's test positive reacting cattle. The agglutination titers of the cow bloods used were always 1:200 or higher.

1. Methods by Which Unknown Clots Were Handled: The unknown blood clots were sent in for culture from the Serology Laboratory after the sera had been removed and agglutination tests for brucellosis and tularemia had been performed. The individual clots were forced through 10 ml syringes into small sterile screw-cap bottles and 1500 units of streptokinase and 15 ml of trypticase-broth were added in the same manner as was done with the rabbit blood. After several days incubation under 2 percent CO_2 tension, part of the supernatants from the "digested" clot cultures was passed through membrane filters. These filters placed on proper medium and the remainder of the supernatants were then incubated under CO_2 tension. The supernatants and digested clots were reincubated in case the first membranes were negative. Second

IV. EXPERIMENTAL RESULTS

A. Organism Growth on Membrane Filters with Double or Single Strength Infusion Media.

The results of the studies described under sections E and F of the Experimental Methods are summarized together in Table I. It can be seen from this table that stock strain #456 Brucella abortus will grow on the surface of the membrane filter by either of the two methods described. The Kruse (agar base) method was superior to the Goetz method (absorbent pad base). Table I also shows that the growth was the same whether the medium used was double or single strength with the Kruse method. Only double strength broth was tried with the Goetz method and this produced light growth. The Kruse agar base technique was the one used throughout the remainder of these studies.

B. Results of Blood Degradation with Distilled Water.

The methodology which produced the following results was given in part 3a. of section G in the Experimental Methods.

Of the four bloods that were treated with distilled water only the supernatant fluids from 3 (whole bloods) were placed on membrane filters.

Results of Filtration: The supernatant from bottle #1 (oxalated blood) plugged the membrane at once; no fluid passed through. The supernatants from bottles #2 (citrated blood) and #3 (citrated blood) passed through only in a small amount (2 to 3 ml) before they too had plugged their respective mem-

TABLE I

Effect of type of medium on growth of *Brucellae* on membrane filters

Method	Plate number	Medium	Appearance of growth on membrane filter
Kruse	1	Double strength agar	Heavy
	2	" " "	"
	3	Single strength agar	"
	4	" " "	"
Goetz	5	Double strength broth	Light
	6	" " "	"

branes. It was noticed from the cell residue in the bottom of the bottles that equal volumes of blood and water in the whole blood samples did not result in complete hemolysis of the samples.

Membrane Filter Colony Counts after Seventy-two hours:
Bottle #2 (10^{-4} dilution of Brucella organisms)...0 Colonies
Bottle #3 (10^{-8} dilution of Brucella organisms)...0 Colonies

C. Results of Blood Degradation with Dilute Acids.

In part 3b. of Section G, a description was given of attempts to break down clotted blood with dilute acids.

Results of Filtration: A few mls of the supernatant from bottle #5 (10^{-6}), treated with 3 percent acetic acid was placed on a membrane filter under suction and it plugged the membrane almost immediately. The supernatant from bottle #6 (10^{-8}), treated with 0.1 N. HCl, behaved in the same manner.

Membrane Filter Colony Counts after Seventy-Two Hours:
Bottle #5 (10^{-6} dilution of Brucella organisms)...0 Colonies
Bottle #6 (10^{-8} dilution of Brucella organisms)...0 Colonies

The clot masses on these dilute acid treated samples hadn't disintegrated much and were black and viscous in appearance.

D. Control Plate Counts of Brucella Organism Suspensions.

The results of the pour plates (prepared in part I, section G) to determine the numbers of Brucella organisms present in the dilutions used in these studies are as follows:

10^{-4} dilution
2000+ colonies/.05 ml

10^{-6} dilution
11 colonies/.05 ml

10^{-8} dilution
0 colonies/.05 ml

The above were counted after seventy-two hours incubation.

E. Results of Blood Degradation with Sand, Glass Beads and Distilled water. The use of abrasive agents with distilled water was described in detail in part 3c. of section G under Materials and Methods.

Results of Filtration: About 5 ml of the supernatant of bottle #1 (citrated cells) passed through the membrane. It passed through the filter at about the same speed as a Seitz filtration and took 30 minutes. Bottle #4 (clotted blood) supernatant was not filtered for time reasons. Bottle #3 (citrated) and #5 (clotted) which were placed in the deep freeze (to determine the effect of freezing on cells as a means of breaking them down for filtration) had a gelatinous, mushy consistency after thawing and were considered unsuitable for filtration. Bottles #2 (citrated) and #6 (clotted) which were being saved for future experiments were not used. It was considered best to use fresh blood with the streptokinase experiments which followed these studies.

Membrane Filter Colony Count after Seventy-two Hours:

Bottle #1 (dilution #F (10^{-8}) of Brucella org.)...0 colonies

F. Control Plate Counts of Brucella Organism Suspensions.

A second set of pour plate controls was set up to determine the number of organisms present in the dilutions

used in the blood-sand-water degradation studies of part 3c. under section G. The numbers of colonies after 72 hours of incubation were as follows:

Dilution #E (10^{-6})	Dilution #F (10^{-8})
One million +/0.1 ml	100,000 +/0.1 ml

G. Results of Blood Clot Degradation with Streptokinase.

The addition of streptokinase to fresh rabbit blood clots was presented as the final method of blood degradation tried in these experiments. The details were given in part 3d. of section G.

Results of Filtration: The supernatant fluids from these clot-enzyme-broth mixtures passed through the membranes instantaneously. This is in direct contrast to all filtrations previously attempted. Previously much difficulty was experienced in trying to filter the supernatants resulting from the physical methods (water, sand, and glass beads) used for breaking up the clots. The clots themselves, appeared to be mostly broken up by reason of the heavy red cell sediment (not hemolyzed) surrounding the clot mass. Shaking of the bottles produced many small chunky pieces. This indicated that the main body of the clot had been acted upon moderately well.

Membrane Filter Colony Counts after 48 hours incubation.

Bottle #1 (Contained 10^{-1} Dilution of Brucella Organisms)
Heavy mass of growth on whole area of membrane.

Bottle #2 (Contained 10^{-2} Dilution of Brucella Organisms)
Moderately heavy mass of growth on whole area of membrane.

Bottle #3 (Contained 10^{-4} Dilution of Brucella Organisms)
Moderately heavy mass of growth on whole area of membrane.

Bottle #4 (Contained 10^{-6} Dilution of Brucella Organisms)
0 Colonies

Bottle #5 (Contained 10^{-8} Dilution of Brucella Organisms)
0 Colonies

Gram stains of the growth on membranes #1, 2 and 3 showed pure Brucella.

From these membrane filter colony counts it can be seen that the recovery of these organisms from entrapment in rabbit blood clots was successful.

H. Results of Controls Set Up in Streptokinase-Blood Studies. In the description of the method of use of streptokinase in part 3d. of section G it was stated that appropriate controls on all phases were set up. It was stated in this manner in order that a description of all the controls used would not have to be given and thereby unnecessarily extend the length of the Materials and Methods. Instead, a brief description of each control and its results will be presented here:

1. Brucella Suspension Control Results.

One tenth ml of each of the 5 dilutions of organisms that were added to the fresh rabbit blood were also placed separately in standard petri dishes and overlaid with liver infusion agar in pour plate fashion. The pour plate suspension counts are as follows:

Dilution	10^{-1}	10^{-2}	10^{-4}	10^{-6}	10^{-8}
Colonies	41	100	62	15	2

Gram stains of the above plates showed colonies of pure Brucella with the exception of 10^{-8} dilution, the colonies of

which were Staphylococci. The above dilutions counts showed Brucella organisms to be present in all suspensions prepared except 10^{-8}.

2. Clot-Enzyme-Broth Control Results.

A single capillary pipetteful (roughly 0.5 ml) from each of the slightly cloudy supernatants (after incubation) of the clot-enzyme-broth mixtures was placed in standard petri dishes and overlaid with liver infusion agar. This was done as a check on the membrane cultures in case the organisms were freed from the clot by the streptokinase action. The results of the pour plate counts after 48 hours incubation are as follows:

Bottle #1
Heavy growth throughout medium
Gram stain: pure Brucella

Bottle #2
Heavy growth throughout
Gram stain: pure Brucella

Bottle #3
300+ Colonies
Gram stain: pure Brucella

Bottle #4
0 Colonies

Bottle #5
0 Colonies

The above pour plate counts agree quite well with the membrane cultures which were taken from the same clot-enzyme-broth source. (Refer to Results, Section G).

3. Streptokinase Growth Inhibition Control.

A 15 ml tube of trypticase-soy broth was inoculated with Brucella from an 72 hour trypticase-soy agar slant and then 1500 units of streptokinase were added and mixed into the suspension. This was the unitage of streptokinase that was added to defibrinate the Brucella-containing rabbit blood clots. The 48 hour results are as follows:

Good growth was obtained in the broth, indicating no apparent inhibition of growth of the Brucella by the enzyme.

I. Results of Brucella Isolation from "Unknown" Human and Cow Bloods. The methodology by which these suspected Brucella-containing clots was handled was given in detail in part 1. under section H of the Materials and Methods. Tables summarizing the findings from these unknowns are presented. From an examination of Table II it is apparent that nearly all of the bloods examined (except #F) had a demonstrable Brucella titer. The P. tularensis titers are presumed to be of a cross reaction type of agglutination. The length of time from when the blood clot was treated with streptokinase to when the first membrane was set up varied from 2 to 21 days. The average time for this period of broth incubation was approximately 9 days.

There were two specimens which showed growth on the first membrane filters. These were #E and #G. Gram stain of #E showed a small gram negative bacillus. On a T.S.I.* slant it gave a reaction typical of an Alcaligenes species. Gram stain of #G showed a Staphylococcus species.

Growth was obtained on four of the first specimens filtered from the supernatants of the unknowns in Table III. The growth of specimen #1 was found to be a Staphylococcus species. Gram stain of specimen #J showed the growth to be a gram positive bacillus in both the first and second membrane cultures. A gram positive bacillus was also the

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* Triple Sugar Iron Medium

TABLE II

Results of membrane filter culture of human bloods

Research No.	A	B	C	D	E	F	G
Serology No.	286	336	385	842	129	196	929
Date Blood Set Up	12-5	12-13	12-13	12-21	12-30	1-4	1-14
Brucella Titer	1:25	1:50	1:25	1:100	1:400	Neg.	1:50
P. tular. Titer	Neg.	Neg.	Neg.	Neg.	Neg.	1:25	1:100
1st Membrane Set Up	12-7	12-16	12-16	1-14	1-14	1-14	1-28
1st Membrane Results	Neg.	Neg.	Neg.	Neg.	Growth	Neg.	Growth
2nd Membrane Set Up	12-16	1-14	1-14	-	-	-	-
2nd Membrane Results	Neg.	Neg.	Neg.	-	-	-	-

TABLE III

Results of membrane filter
culture of human and cow bloods

Research No.	H	I	J	K	L	M
Serology No.	752	308	145	304	742	Cow #6
Date Blood Set Up	1-14	1-14	1-14	1-16	1-28	2-18
Brucella Titer	1:50	1:50	1:50	Neg.	1:400	1:200
P.tular. Titer	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
1st Membrane Set Up	1-28	1-28	1-28	1-28	2-18	2-29
1st Membrane Results	Neg.	Growth	Growth	Neg.	Growth	Growth
2nd Membrane Set Up	-	-	2-18	2-18	-	-
2nd Membrane Results	-	-	Growth	Growth	-	-

cause of growth on the second membrane in specimen #K. In both #1 and #M (cow) in Table III the organisms were gram negative bacilli which on T.S.I. slants proved to be coliform bacilli.

In the membrane results tabulated in Table IV there was less evidence of outside contamination than in the growth results of Table III. The one membrane (#O) in Table IV which produced growth was shown by Gram's stain to be a Staphylococcus species. The length of incubation time for the broth-clot mixtures in Table IV was more uniform than in either Tables III or II. It was 11 days in 5 out of the 6 clots cultured.

In Table V one can see that 4 out of the 5 bloods cultured were from cows. Growth was also obtained on all of these cow blood clots. Gram negative bacilli were responsible for the growth on the membranes of all these specimens (#U, V, W, & X). The T.S.I. reaction of all these bacilli was of the coliform type.

TABLE IV

Results of membrane filter culture of human bloods

Research No.	N	O	P	Q	R	S
Serology No.	523	564	987	824	623	810
Date Blood Set Up	2-18	2-18	2-18	2-18	2-18	2-29
Brucella titer	1:200	1:50	1:100	1:50	1:200	1:50
P.tular. titer	Neg.	1:25	1:25	1:100	Neg.	Neg.
1st Membrane Set Up	2-29	2-29	2-29	2-29	2-29	3-3
1st Membrane Results	Neg.	Growth	Neg.	Neg.	Neg.	Neg.

TABLE V

Results of membrane filter culture of human and cow bloods

Research No.	T	U	V	W	X
Serology No.	949	Cow #5	Cow #6	Cow #8	Cow #10
Date Blood Set Up	2-29	2-29	2-29	2-29	2-29
Brucella Titer	1:100	1:200	1:200	1:200	1:200
P.tular. Titer	-	-	-	-	-
1st Membrane Set Up	3-3	3-3	3-3	3-3	3-3
1st Membrane Results	Neg.	Growth	Growth	Growth	Growth

V. DISCUSSION

The fact that the membrane filter will support the growth of a fastidious organism like Brucella abortus was shown in these studies, confirming the work of Braun and Kelsh (18), although these findings were arrived at before Braun and Kelsh (18) published their data.

An agar base (27) nutrient for the membrane disc was also found by this author to support the growth of Brucella organisms better than an absorbent pad (16). This was in agreement with Braun and Kelsh (18), but not so with Tidwell and Gee (21) who used the pads for a nutrient source. Tidwell and Gee (21) however, grew other organisms that are not considered as fastidious as Brucella. The concentration of this agar base medium for the organisms used was also found to give better results in these experiments in single strength than in double strength as suggested by Kruse (27).

The physical methods tried for the degradation of the blood clots to prepare them for filtering were of no avail in these studies. Even with citrated blood, the filtration was slow and tedious which seemed to defeat the premise of finding an efficient and easy method. Braun and Kelsh (18) admit in their methods that it took them 30 minutes to filter 1 ml of laked heparinized rabbit blood. Tidwell and Gee (21) claim their blood aliquot (2 ml) filters faster (in 5 minutes) than Brauns, but they spend 80 minutes laking and washing

their heparinized specimens.

The defibrination of the blood clots in these studies with streptokinase would seem to multiply the time factor, but the author feels that the Brucellae would in the long run be much better off being gently released by enzyme action than beaten out of their very secure blood clot stronghold.

The preliminary experiments in these studies would have been strengthened if a rabbit had been actually infected and then the reisolation of the Brucellae attempted with the streptokinase technique before the patient and animal unknown bloods were set up with this method. This was actually tried but the rabbit failed to become infected with the laboratory stock strain. Because of this failure it was not mentioned in the materials and methods section.

Another preliminary procedure that would have helped in these studies would have been to have tried various unitages (high and low) of streptokinase against a known virulent Brucella species as a better streptokinase toxicity control.

It was of interest to isolate organisms by this method from the unknown blood clots, but disappointing when none of the gram negative bacilli turned out to be Brucella. Even though the cow bloods had high Brucella agglutination test titers, they were all so heavily contaminated with E. coli before reaching this laboratory that any Brucella

present would have been overgrown. The addition of crystal violet in high dilution to the streptokinase-trypticase-soy broth was apparently quite effective in holding down the gram positive contamination that so easily enters into the blood-letting and handling processes, but the gram negative type of contaminant posed a different problem. Future studies should definitely consider this problem.

VI. SUMMARY

It was found that the laboratory stock strain (#456) of Brucella abortus used in these studies will grow on the surface of the membrane filter disc. A single strength concentration of ingredients in an agar base medium appeared to be the best method of supplying nutrients to this organism when being cultured on the filter.

The results of whole and clotted blood degradation with distilled water, dilute acids, and sand-glass beads-distilled water were found to be poor when attempts to filter the supernatants from these methods were made. There was also no reisolation of artificially inoculated Brucella organisms made from the membranes cultured from these techniques.

The fibrinolytic dissolving of rabbit blood clots with the streptococcal enzyme, streptokinase, was found to produce excellent results in both digesting the clot and producing a supernatant that would pass through the membrane filter very rapidly. Recovery of Brucella organisms artificially inoculated into the blood clots was made. Quantitative estimation of the number of organisms inoculated could not be made from the number reisolated because of prolonged incubation of the digesting clots.

This streptokinase Brucella isolation technique was applied to unknown animal and human bloods suspected of harboring the organisms of brucellosis. No positive isola-

tions of Brucella were made. A variety of organisms were isolated, most of which were probably contaminants introduced into the blood before being received at this laboratory.

Crystal violet seemed to prevent excessive gram positive organism contamination, but methods for preventing gram negative organism contamination without destroying the Brucella should be sought in future studies.

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AN IMPROVED METHOD OF ISOLATING BRUCELLOSIS ORGANISMS FROM BLOOD CLOTS UTILIZING THE MEMBRANE FILTER

by

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The Millipore type of membrane filter was used to culture a Brucella abortus species from artificially infected rabbit blood clots. Attempts were made to isolate naturally occurring species of these organisms from both human and animal bloods on this filter.

The blood clots were treated with both physical and enzymatic methods in an attempt to prepare a liquid that would pass through the membrane filter. The results of blood degradation with distilled water, dilute acids and sand-glass beads-distilled water were found to be poor when attempts were made to filter the supernatants prepared by these methods. There was no reisolation when the clot was artificially inoculated with Brucella species when treated by physical and chemical methods. The use of fibrinolysin (streptokinase) to dissolve the rabbit blood clots produced excellent results. Both the digestion of the clot and the production of a filterable supernatant that would pass through the membrane were obtained. Recovery of the Brucella species artificially inoculated into these enzymatically treated clots was made.

No positive isolations of Brucella species were made when this streptokinase Brucella isolation technique was applied to unknown animal and human bloods suspected of harboring this organism. A variety of organisms were isolated, most of which were probably contaminants introduced into the blood before being received at this laboratory.